PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 21/00, A61K 31/70	A1	(11) International Publication Number: WO 97/29116
		(43) International Publication Date: 14 August 1997 (14.08.97)

(21) International Application Number: PCT/GB97/00327

(22) International Filing Date: 6 February 1997 (06.02.97)

(30) Priority Data:
9602326.2 6 February 1996 (06.02.96) GB

(71) Applicant *(for all designated States except US)*: CRUACHEM LIMITED [GB/GB]; Todd Campus, West of Scotland Science Park, Acre Road, Glasgow G20 0UA (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: REESE, Colin, Bernard [GB/GB]; 21 Rozel Road, Clapham, London SW4 0EY (GB). RAO, Mothe, Vaman [IN/US]; 826 Charlestown Meadows Drive, Westborough, MA 01581 (US).

(74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).

(81) Designated States: DE, ES, GB, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SULPHUR CONTAINING DINUCLEOTIDE PHOSPHORAMIDITES

(57) Abstract

There is provided a process for the solid phase synthesis of phosphorothioate oligonucleotides in which a dimeric phosphoramidite synthon is used to extend the oligonucleotide chain, the synthon having an optionally protected thioester group in its internucleotide linkage. Novel dimeric phosphoramidite synthons having such a thioester group are also described. The process enables increased yield of the oligonucleotide of interest with enhanced separation from impurities. The presence of the thioester linkage stabilises the oligonucleotide end product, facilitating its use as an anti-sense oligonucleotide analogue for gene therapy.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

SULPHUR CONTAINING DINUCLEOTIDE PHOSPHORAMIDITES

The present invention relates to dinucleotide phosphoramidites having a non-bridging sulphur group attached to the phosphorus moiety, the synthesis of these compounds and their use in the synthesis of phosphorothioate oligonucleotides.

The standard methodology for oligonucleotide synthesis relies upon solid phase chemistry. In a typical synthetic protocol phosphoramidites are added in a stepwise manner to an initial immobilised nucleoside, with protecting and deprotecting steps as necessary in each cycle. The process is now automated and is normally able to produce 10^{-6} mol quantities of the desired end product. A suitable methodology is described by Beaucage in Methods in Molecular Biology, Vol 20, Protocols for Oligonucleotides and Analogues, ed Agrawal, Humana Press, Totawa, 1993, pages 33-61.

More recently, the synthesis of S-alkyl esters of 2'-deoxyribonucleoside 3'-phosphorothioates has been reported (see Liu et al, J. Chem. Soc. Perkin Trans 1 : 1685-1694 (1995)) and the use of such compounds in the synthesis of oligonucleotide phosphorothioates was

suggested.

Phosphorothioate oligonucleotides are regarded as the first generation of antisense oligonucleotide analogues which have been successfully tested *in vitro* and *in vivo* as inhibitors of gene expression (see, "Oligonucleotides: Antisense Inhibitors of Gene Expression", Ed. Cohen, Macmillan, London, 1989 and "Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS", Ed. Wickstrom, Wiley-Liss, New York, 1992). At present, a few uniformly modified phosphorothioate oligonucleotides are in human clinical trials and have the potential to be used as approved drugs. (see, Ravikumar *et al*, Bioorganic & Medicinal Chemistry Lett.: 2017-2022 [1994]). Large quantities, multiple gram to multiple kilogram, of high purity phosphorothioate oligonucleotides are required at low and acceptable cost suitable for therapeutic applications.

Phosphorothioate oligonucleotides are isoelectronic analogues of natural oligonucleotides in which one of the non-bridging internucleotide oxygen atoms is replaced by a sulphur atom. The solid phase synthesis of phosphorothiate oligonucleotides has been achieved using H-phosphonate chemistry (see, Froehler *et al*, Tetrahedron Lett. 5575-5578 [1986]) where only one sulphur transfer step is required after assembling the desired sequence to convert all the internucleotide linkages to phosphorothioates, or the phosphoramidite approach (see, Stec *at al*, J. Am. Chem. Soc., 6077-6079 [1984] and Rao *et al*, Tetrahedron Lett., 6741-6744 [1994]) where monomeric phosphoramidites are added in each synthetic cycle and a stepwise sulphurisation instead of iodine oxidation step in an otherwise standard synthetic cycle is used to assemble the

desired phosphorothioate oligonucleotides. The solid phase monomeric phosphoramidite chemistry is routinely used to synthesize phosphorothioate oligonucleotides (on micromole to millimole scale) as considerable efforts have been expended in enhancing the efficiency of the synthesis such as (i) the use of improved synthetic cycle protocols and solid supports (see, Ravikumar *et al*, Bioorganic & Medical Chemistry Lett., 2017 [1994]) (ii) sulphur transfer reagents (see Rao *et al*, Tetrahedron Lett., 6741 (1994) and references cited therein), (iii) capping and deblocking reagents (see, Agrawal *et al*, Tetrahedron Lett., 8565 [1994]). However, problems still remain both in terms of consistent yields and quality of the final oligonucleotide phosphorothioate. In particular the n-1 and n+1 impurities are very similar to the full length product "n" and vary from batch to batch, especially when reduced excesses of monomeric nucleoside phosphoramidite synthons are used in each synthetic cycle. In order to meet the quality specifications of the full length phosphorothioate oligonucleotide needed for therapeutic applications, which are very high, it is necessary to repeatedly purify the product, free from n-1 and n+1 impurities. Consequently the process will result in lowering the yield of the full length product and hence the overall process might not be cost effective.

Whilst the potential utility of phosphorothioates has been recognised there still remains a need for an effective and efficient manufacture of these complex molecules. In particular it has not previously been recognised that dimeric or larger phosphoramidite blockmers could be advantageously applied in their synthesis via solid phase chemistry.

In order to alleviate some of these problems, recent efforts have been focused on investigating the feasibility of the large scale synthesis of phosphorothioate oligonucleotides by the phosphotriester approach in solution (see Reese *et al*, J. Chem. Soc. Perkin Trans., 1685 [1995] and Imbach *et al*, Antisense Res. Dev. 39 [1995]. While this approach offers definite advantages over the solid phase monomeric phosphoramidite chemistry, in that:

(i) it is more suitable for scale-up for synthesis in much larger quantities, (e.g. millimoles to mole + scale)

(ii) it allows addition of two or more nucleotide residues at a time (i.e., block synthesis)

(iii) it offers the choice of purifying fully protected blockmers at different stages prior to assembling the desired sequence and

(iv) it allows much easier purification of the final product,

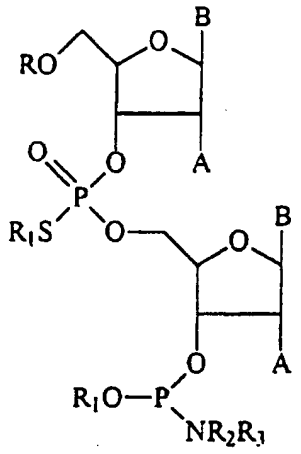
it requires further development.

However, the solid phase phosphoramidite approach (useful for micromole to millimole scale synthesis) can be improved by the addition of a dimeric phosphoramidite synthon instead of a monomeric phosphoramidite synthon during the synthetic cycle and this forms the basis of the present invention. The dimeric phosphoramidite approach would achieve an increased yield (as the number of steps required to produce a particular oligonucleotide will be reduced) and enhanced separation of the desired oligonucleotide

from the impurities (as their use results in n-2 and n+2 impurities instead of n-1 and n+1 impurities) due to the greater difference in size.

The present invention provides an improved process for the solid phase synthesis of phosphorothioate oligonucleotides using dinucleotide phosphoramidite synthons containing the S-protected phosphorothioate ester internucleotide linkage and a 3'-phosphoramidite functional group.

The present invention provides novel compounds of formula I



wherein

B represents a heterocyclic amine base or a derivative thereof;

R represents an acid labile protecting group;

R_1 represents a protecting group, preferably selected from the group consisting of 2-cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl and 4-nitrophenyl;

R_2 represents a blocking or protecting group;

R_3 represents a blocking or protecting group; and

A represents a hydrogen atom, or an alkoxy, allyloxy or suitably protected hydroxy group.

The dinucleotide phosphoramidite of formula I can be used in conventional automated solid phase synthesis to produce phosphorothioate oligonucleotides.

Thus, the present invention also provides a process for producing an oligonucleotide having at least one phosphorothioate linkage, said process comprising providing a compound of formula I above for reaction with the terminal nucleoside of the nucleotide chain located at the solid phase to assemble the nucleotide chain. As used herein the term "nucleotide chain" includes a single nucleoside located at the solid phase which will itself be the terminal group available for reaction.

Group R is desirably 4,4'-dimethoxytrityl, but any other suitable protecting group may also be used.

Groups R_2 and R_3 may each independently be an alkyl or aryl group.

The heterocyclic base of group B may be, for example a purine, such as adenine, guanine or derivatives thereof, or a pyrimidine, such as cytosine, uracil, thymine or derivatives thereof. As derivatives may be mentioned alkylated derivatives (especially methylated derivatives) and halogenated derivatives, but are not specially limited thereto. Uracil and derivatives thereof may be especially convenient for use.

The present invention will now be further described with reference to the following non-limiting Examples.

Example 1a

Triethylammonium salt of 5'-O-(4,4'-dimethoxytrityl)thymidine S-(2-cyanoethyl) 3'-phosphorothioate (see Reese et al, J. Chem. Soc. Perkin Trans. 1: 1685 [1995])

To a stirred solution of 1,2,4-triazole (8.28g, 0.126 mol) in anhydrous tetrahydrofuran (250ml) was added triethylamine (18.08ml, 0.13 mol) and phosphorus trichloride (3.5ml, 40 mmol) at approximately -35°C (methanol-CO_2 bath). The reaction was stirred for 15 minutes, after which 5'-O-(4,4'dimethoxytrityl) thymidine (5.546g, 10.2 mmol) in tetrahydrofuran (200ml) was added. After a further 30 minutes, triethylamine - water (60ml, 1:1 v/v) was added dropwise with stirring and the reaction mixture was allowed to warm up to ambient temperature. The solvent was removed under reduced pressure. The residue was dissolved in chloroform (500ml) and washed with 0.5M triethylammonium bicarbonate (2 x 250ml). The organic layer was dried ($MgSO_4$) and evaporated. The residue was co-evaporated with acetonitrile (3 x 100ml), and then dissolved in anhydrous dichloromethane (180ml). N-(2-Cyanoethylthio)phthalimide (3.09g, 13.3 mmol) was added, followed by N-methylmorpholine (6.67ml, 60 mmol) and chlorotrimethylsilane (5.07ml, 40 mmol). The mixture was allowed to stir at ambient temperature. After 3 hours, the reaction mixture was poured into 0.5M triethylammonium bicarbonate (200ml). The organic layer was separated and the aqueous layer was extracted with dichloromethane (200ml). The combined organic layers (dried over $MgSO_4$) were evaporated. The residue was purified by short-column chromatography and the product-containing fractions, which were eluted with $CHCl_3$-MeOH (90:10 to 85:15 v/v), were evaporated under

reduced pressure. The residue was dissolved in chloroform (40ml) and the title compound was obtained by precipitation from petroleum ether (b.p. 30-40°C, 400ml) as a colourless solid (8.10g).

δ_H $[CD_3)_2SO]$: 1.18 (1.5 H, t, J = 7.3 Hz), 1.36 (3 H, s), 2.40 (2 H, m), 2.69 (2 H, m), 2.83 (2 H, m), 3.03 (1 H, q, J = 7.2 Hz), 3.17 (1 H, m), 3.32 (1 H, m), 3.74 (6 H, s), 4.19 (1 H, m), 4.91 (1 H, m), 6.23 (1 H, t, J = 7.2 Hz), 6.89 - 7.41 (13 H, m), 7.52 (1 H, s) 11.40 (1 H, s).

δ_P $[CD_3)_2SO]$: 13.9 ppm

HPLC data: R_1 = 9.65 minute (Programme 1)

Column : ODS 5μ (5 x 250 mm)

Eluting Conditions : Curve Select : linear gradient, time of programme = 10 minutes; flow : 1.5 ml/minute;

Initial conditions: 0.1M triethylammonium acetate (TEAA) buffer : acetonitrile (7:3, v/v)

Final conditions: 0.1M TEAA buffer : acetonitrile (2:8, v/v)

Example 1b

Triethylammonium salt of N-benzoyl-5'-O-(dimethoxytrityl)deoxycytidine S-(2-cyanoethyl) 3'-phosphorothioate

This compound was prepared on the same scale and in precisely the same way as the thymidine derivative described above. N-benzoyl-5'-O-(dimethoxytrityl) deoxycytidine (6.336g, 10 mmol) was converted into the title compound (8.84g) as a colourless solid.

δ_H [$(CD_3)_2SO$]: 1.19 (6 H, t, J = 7.3 Hz), 1.36 (3 H, s), 2.32 (1 H, m), 2.68 (1 H, m), 2.85 (2 H, m), 3.06 (4 H, q, J = 7.3 Hz), 3.41 (2 H, m), 3.75 (6 H, m), 4.29 (1 H, m), 4.85 (1 H, m), 6.18 (1 H, t, J = 6.3 Hz), 6.90 - 8.00 (19 H, m), 8.18 (1 H, d, J = 7.5 Hz) 11.31 (1 H, s).

δ_P [$(CD_3)_2SO$]: 13.2 ppm

HPLC data: R_1 = 11.25 minutes (Programme 1)

Example 1c

5'-O-(Dimethoxytrityl) thymidin-3'-yl-N-benzoyldeoxycytidin-5'-yl S-(2-cyanoethyl) phosphorothioate

A solution of triethylammonium salt of 5'-O-(dimethoxytrityl)thymidine-S-(2-cyanoethyl)-3'-phosphorothioate (2.012g, 2.5 mmol) (from Example 1a), N-benzoyldeoxycytidine (1.035g, 3.125 mmol) and 3-nitro-1,2,4-triazole (0.998g, 8.75 mmol) in pyridine (25 ml) was concentrated to dryness under reduced pressure. This process was repeated twice more and the residue was dissolved in dry pyridine (20ml). Mesitylene-2-sulfonyl chloride (1.64g, 7.5 mmol) was added and the solution was allowed to stir for 30 minutes. The reaction was quenched with saturated aqueous sodium bicarbonate (2.5ml), and the products were partitioned between chloroform (50ml) and saturated aqueous sodium bicarbonate (150ml). The organic layer was separated and the aqueous layer was extracted with chloroform (4 x 30ml). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was co-evaporated with toluene (2 x 20ml) and then purified by short-column chromatography. The appropriate fractions, eluted with $CHCl_3$-MeOH (98:2 to 96.5-3.5 v/v) were combined and evaporated under reduced pressure. A solution of the residue in chloroform (10ml) was added dropwise to petroleum ether (b.p. 30-40°C, 200ml) to give the title compound as a precipitate (1.57g, 61.8%).

δ_H $[CD_3)_2SO]$: 1.45 (3 H, s), 2.15 (1 H, m), 2.35 (1 H, m), 2.57 (2 H, m), 2.90 (2 H, m) 3.10 (2 H, m), 3.31 (2 H, m), 3.73 (6 H, s), 4.07 (1 H, m), 4.23 (2 H, m), 4.32

(2 H, m), 5.23 (1 H, m), 5.56 (1 H, , d, J = 4.3 Hz), 6.16 (1 H, m), 6.25 (1 H, m), 6.87 - 8.00 (20 H, m), 8.15 (1 H, m), 11.27 (1 H, s), 11.41 (1 H, s).

On treatment with D_2O signals at 11.27, 11.41, 5.56 ppm diminished in intensity.

δ_H $[CD_3)_2SO]$; 27.7, 28.0 ppm

HPLC data: R_1 = 12.12 minutes, 12.27 minutes (programme 1)

Example 1d

N-benzoyl-5'-O-(dimethoxytrityl)deoxycytidin-3'-yl thymidin-5'yl S-(2-cyanoethyl) phosphorothioate

A solution of the triethylammonium salt of N-benzoyl-5'-O-(dimethoxytrityl)deoxycytidine S-(2-cyanoethyl) 3'-phosphorothioate (4.42g, 5 mmol) (from Example 1b), thymidine (1.519g, 6.25 mmol) and 3-nitro-1,2,4-triazole (2.00g, 17.5 mmol) in dry pyridine (20ml) was concentrated to dryness under reduced pressure. This process was repeated twice more and the residue was dissolved in dry pyridine (50ml). Mesitylene-2-sulfonyl chloride (3.28g, 15.0 meal) was added and the solution was allowed to stir for 30 minutes. The reaction was quenched with saturated aqueous sodium bicarbonate (me) and the products were partitioned between chloroform (100ml) and 0.5M triethylammonium bicarbonate (200ml). The organic layer was separated and the aqueous layer was extracted with chloroform (3 x 50ml). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was co-evaporated with toluene (3 x 20ml) and then purified by short-column chromatography. The appropriate fractions, eluted with $CHCl_3$-MeOH (98:2 to 97:3 v/v) were combined and evaporated under pressure. A solution of the residue in chloroform (15ml) was added dropwise to petroleum ether (b.p. 30-40°C, 300ml) to give the title compound as a precipitate (3.06g, 60%).

δ_H $[CD_3)_2SO]$: 1.79 (3 H, s), 2.15 (2 H, m), 2.48 (1 H, m), 2.79 (2 H, m), 2.90 (2 H, m) 3.00 (2 H, m), 3.38 (2 H, m), 3.74 (6 H, s), 3.99 (1 H, m), 4.34 (4 H, m), 5.15
(1 H, m), 5.52 (1 H, d, J = 4.5 Hz), 6.19 (2 H, m), 6.89 - 8.03 (20 H, m), 8.18 (1 H, d, J = 7.4 Hz), 11.32

(1 H, z), 11.35 (1 H, s).

On treatment with D_2O signals at 5.52, 11.32 and 11.35 ppm diminished in intensity.

δ_p $[CD_3)_2SO]$; 27.7, 27.9 ppm

HPLC data: R_1 = 13.00 minutes, 13.13 minutes (Programme 1)

Example 2a

5'-O-(Dimethoxytrityl)-thymidin-3'-yl-3'-[(2-S-cyanoethyl)phosphoryl]-5'-N-benzoyl-2'-deoxycytidine-3'-[(2-cyanoethyl)-N,N-diisopropyl] phosphoramidite

Abbreviation: T-P(s)-dC-CEPA

5'-O-(Dimethoxytrityl)thymidin-3'-yl N-benzoyldeoxycytidin-5'-yl S-(2-cyanoethyl) phosphorothioate (8.20g, 8.151 mmol, 1 mol eq) (from Example 1c) was dissolved in dry dichloromethane (AR grade) (120ml) under an argon blanket, and allowed to stir for 5 minutes. To this solution was added diisopropyl-ammonium tetrazolide (1.394g, 1 mol eq) followed by bis-(N,N-diisopropylamino)-(2-O-cyanoethyl) phosphoramidite (4.914g, 2 mol eq) and the reaction mixture allowed to stir under an argon blanket for 1.5 hours. The reaction was then washed with water (75ml), saturated NaCl solution (75ml) and saturated $NaHCO_3$ (75ml). The organic layers were separated and the aqueous layers were back extracted with dichloromethane (25ml) and the extract was added to the organic layers, which were then dried over anhydrous sodium sulphate (50g), filtered and then evaporated to a foam. The foam was then dissolved in dichloromethane (20ml) and purified on a silica chromatography column with a silica/product ration of 10:1. The column was first packed with 1% pyridine in dichloromethane, then once the product had been loaded onto the column it was eluted with dichloromethane (100ml), MeCN (2000ml), and 10% MeOH in dichloromethane (250ml) to strip the column. The appropriate fractions were combined and evaporated under reduced pressure to a foam. The product was then dissolved in dichloromethane (50ml) and added dropwise to pentane (500ml) to give a

precipitate. This was then dissolved in dichloromethane and filtered through a 1 micron filter system, then evaporated to a foam and placed onto a freeze drier for a minimum of 8 hours. Yield = 7.5g, 79.3%. δ_p [$CDCl_3$]: 26.85, 148.91, 149.52 ppm.

Analytical data from the compound formed is presented in Fig 1.

Example 2b

5'-O-(Dimethoxytrityl)-N-benzoyl-2'-deoxycytidine-3'-yl-3'-[2-S-cyanoethyl) phosphoryl]-5'-thymidine-3'-[2-cyanoethyl)-N,N-diisopropyl] phosphoramidite

Abbreviation: dC-P(S)-T-CEPA

5'-O-(Dimethoxytrityl)-N-benzoyl-deoxycytidin-3'-yl thymidin-5'-yl S-(2-cyanoethyl) phosphorothioate (8.00g, 7.952 mmol, 1 mol eq) (from Example 1d) was dissolved in dry dichloromethane (AR grade) (120ml) under an argon blanket and allowed to stir for 5 minutes. To this solution was added diisopropylammonium tetrazolide (1.36g, 1 mol eq) followed by bis(N,N-diisopropyl-amino)-(2-O-cyanoethyl) phosphoramidite (4.794g, 2 mol eq), and the reaction mixture was allowed to stir under an argon blanket for 1.5 hours. The reaction was then washed with water (75ml), saturated NaCl solution (75ml). The organic layers were separated and the aqueous layers were back extracted with dichloromethane (25ml) and the extract was added to the organic layers, which were then dried over anhydrous sodium sulphate (50g), filtered, and then evaporated to a foam. The foam was then dissolved in dichloromethane (20ml) and purified on a silica chromatography column with a silica/product ratio of 10:1. The column was first packed with 1% pyridine in dichloromethane, then once the product had been loaded onto the column it was eluted with dichloromethane (100ml), MeCN (1000ml), and 10% MeOH in dichloromethane (250ml) to strip the column. The appropriate fractions were combined and evaporated under reduced pressure to a foam. The product was then dissolved in dichloromethane (50ml) and added dropwise to pentane (500ml) to give a precipitate. This was then dissolved

in dichloromethane and filtered through a 1 micron filter system, then evaporated to a foam and placed onto a freeze drier for a minimum of 8 hours. Yield = 7.00g 73.0%. δ_p [$CDCl_3$]: 26.83, 149.09, 149.23 ppm

Example 3

Automated solid-phase synthesis of phosphorothioate oligonucleotides

Synthesis of phosphorothioate oligonucleotides were carried out using a Cruachem PS250 DNA/RNA synthesizer. Cruachem standard DNA phosphoramidites and reagents were used unless otherwise stated. One μm phosphorothioate synthetic cycle protocol in conjunction with a solution of 0.05M Beaucage reagent [3H-1,2-benzodithiol-3-one-1,1-dioxide] with 60 seconds reaction time for thiolation was used.

To evaluate the potential use of the present invention for the synthesis of phosphorothioate oligonucleotides, stringent coupling reaction conditions on the use of phosphoramidite synthons (3-4 excess molar equivalents) in conjunction with controlled pore glass containing a higher nucleoside loading (100 μm/gram) were used. The compounds formed in Examples (2a) and (2b) were used as the corresponding solutions in anhydrous CH_3CN (0.1M).

To demonstrate the improvements of the present invention, a few phosphorothioate oligonucleotides were synthesized using the monomeric phosphoramidite synthons and the aforesaid conditions. Identical phosphorothioate oligonucleotide sequences were synthesized using the dimeric phosphoramidite synthons and after appropriate deprotection steps, the resulting oligonucleotides were compared.

Oligonucleotide sequences:

Seq ID Nos 1 & 4 : $(TC)_{10}T$ - 21 mer
Seq ID Nos 2 & 5 : $(CT)_{10}T$ - 21 mer
Seq ID Nos 3 & 6 : TCC TTC TCT CCT CTC TTC CTA - 21 mer

Synthesis of Seq ID Nos 1-3

The Sequences were produced using monomeric phosphoramidite synthons. The synthesis protocol therefore required 20 synthesis cycles and 20 sulphurisation steps.

*ACE = > 98%
(based on DMT cation assay)

Synthesis of Seq ID Nos 4-6

The Sequences were produced using the dimeric phosphoramidite synthons (T-P(s)-dc-CEPA and dc-p(s)-T-CEPA). The synthesis protocol therefore required 10 synthesis cycles and 10 sulphurisation steps.

*ACE = > 98%
(based on DMT cation assay)

* Average coupling efficiency

Deprotection of Oligonucleotide Sequences:

(a) Seq ID Nos 1 to 3 synthesized using monomeric phosphoramidite synthons were released from the solid support and deprotected by treating with concentrated aqueous ammonia (1.0mL) at 55°C for 12 hours. The ammoniacal solution was evaporated to a pellet under reduced pressure and the unpurified (crude) oligonucleotides were analysed.

oligonucleotide with anhydrous pyridine (1.0 mL) using vacuum centrifugation. Once dried, the material was treated with a solution of DBU (1,8-Diazabicyclo[5, 4,0]-undec-7-ene) in anhydrous pyridine (5:95, v/v 1.0ml) for 2 hours at 30°C. The solvents were then removed and the residue was then treated with concentrated aqueous ammonia (1.0ml) at 55°C for 12 hours. The ammoniacal solution was evaporated to a pellet under reduced pressure and the unpurified (crude) oligonucleotides were analysed.

HPLC (Ion Exchange) analysis:

Ion-exchange HPLC analysis of phosphorothioate oligodeoxy-nucleotides was carried out using a Gilson 712 Gradient system with dual pumps and fitted with a Gilson 117 UV Detector (280nm). A 5 micron Nucleopac PA100 column (5 x 250 mm) was used with eluents [A] : 20 mM Tris-HCl buffer, pH = 8.0 and [B] : 400 mM sodium perchlorate in buffer [A].

The results are shown in Figs 2 to 4.

Fig 2 shows a comparison of anion-exchange (NucleoPac PA-100) chromatograms of unpurified 5'-O-DMT-on phosphorothioate oligomers $(TC)_{10}T$ 21-mer (Seq 1D Nos 1 and 4). Fig 2A gives the results for the 21-mer synthesised with monomeric phosphoramidites (Seq 1D No 1) which has a product purity of 68.5%. Fig 2B gives the results for the 21-mer synthesised with dimeric phosphoramidites (Seq 1D No 4) which has an increased product purity of 78.0%.

Fig 3 shows a comparison of anion-exchange (NucleoPac PA-100) chromatograms of unpurified 5'-O-DMT-on

product purity of 78.0%.

Fig 3 shows a comparison of anion-exchange (NucleoPac PA-100) chromatograms of unpurified 5'-O-DMT-on phosphorothioate oligomers $(CT)_{10}A$ 21-mer (Seq ID Nos 2 and 5). Fig 3A gives the results for the 21-mer synthesised with monomeric phosphoramidites Seq ID No 2) which have a product purity of 74.0%. Fig 3B gives the results for the 21-mer synthesised with dimeric phosphoramidites (Seq ID No 5) which has an increased product purity of 83.0%.

Fig 4 shows a comparison of anion-exchange (NucleoPac PA-100) chromatograms of unpurified 5'-O-DMT-on phosphorothioate oligomers (TCC TTC TCT CCT CTC TTC CTA) 21-mer (Seq ID Nos 3 and 6). Fig 4A gives the results for the 21-mer synthesised with monomeric phosphoramidites (Seq ID No 3) which have a product purity of 73.8%. Fig 4B gives the results for the 21-mer synthesised with dimeric phosphoramidites (Seq ID No 6) which has an increased product purity of 85.5%.

Fig 5 is a comparison of ^{31}P NMR spectra of unpurified 5'-O-DMT-on phosphorothioate oligomers for Seq ID Nos 3 and 6.

A: synthesised using monomeric phosphoramidites (Seq ID No 3)

B: synthesised using S-dimeric phosphoramidites (Seq ID No 6).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: CRUACHEM LTD
(B) STREET: WEST OF SCOTLAND SCIENCE PARK, TODD CAMPUS, ACRE ROAD
(C) CITY: GLASGOW
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): G20 0UA

(ii) TITLE OF INVENTION: COMPOUNDS

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: GB 9602326.2

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTCTCTCTC TCTCTCTCTC T 21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTCTCTCTCT CTCTCTCTCT A 21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCCTTCTCTC CTCTCTTCCT A 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)
(D) OTHER INFORMATION:/mod_base= OTHER
/label= PHOSPHOROTHIOAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCTCTCTCTC TCTCTCTCTC T 21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)
(D) OTHER INFORMATION:/mod_base= OTHER
/label= PHOSPHOROTHIOAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTCTCTCTCT CTCTCTCTCT A 21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)
(D) OTHER INFORMATION:/mod_base= OTHER
/label= PHOSPHOROTHIOAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCCTTCTCTC CTCTCTTCCT A 21

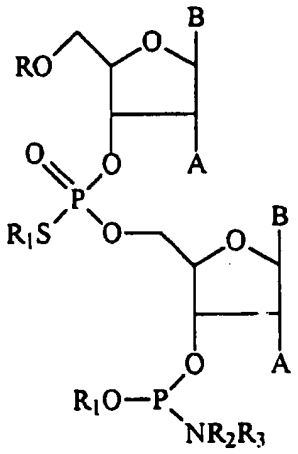
CLAIMS

1. A process for the solid phase synthesis of phosphorothioate oligonucleotides, said process comprising the addition of at least one dimeric phosphoramidite synthon during the synthetic cycle, wherein said dimeric phosphoramidite synthon comprises in its internucleotide linkage an optionally protected thioester group.

2. A process as claimed in Claim 1 wherein said dimeric phosphoramidite synthons are used as reactants in each synthetic cycle.

3. A process as claimed in either one of Claims 1 and 2 wherein said thioester group present in said internucleotide linkage is protected by a 2-cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl or 4-nitrophenyl group.

4. A dimeric phosphoramidite synthon being a compound of Formula I:



wherein
B represents a heterocyclic amine base or a derivative thereof;
R represents an acid labile protecting group;
R_1 represents a protecting group;
R_2 represents a blocking or protecting group;
R_3 represents a hydrogen atom, or an alkoxy, allyloxy or suitably protected hydroxy group.

5. A compound as claimed in Claim 4 wherein group B is an adenine, guanine, cytosine, uracil or thymine base or the alkylated or halogenated derivatives of any of those bases.

6. A compound as claimed in Claim 5 wherein at least one group B is uracil or methylated uracil.

7. A compound as claimed in any one of Claims 4 to 6 wherein group R is a 4,4'-dimethoxytrityl group.

8. A compound as claimed in any one of Claims 4 to 7 wherein each group R_1 is independently a 2-cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl or 4-nitrophenyl group.

9. A compound as claimed in any one of Claims 4 to 8 wherein each group R_2 and group R_3 is independently an alkyl or aryl group.

10. Use of a compound as claimed in any one of Claims 4 to 9 in the synthesis of phosphorothioate oligonucleotides.

11. Use of phosphorothioate oligonucleotides produced in accordance with the process of Claims 1 to 3 as anti-sense nucleotides for inhibition of gene

expression.

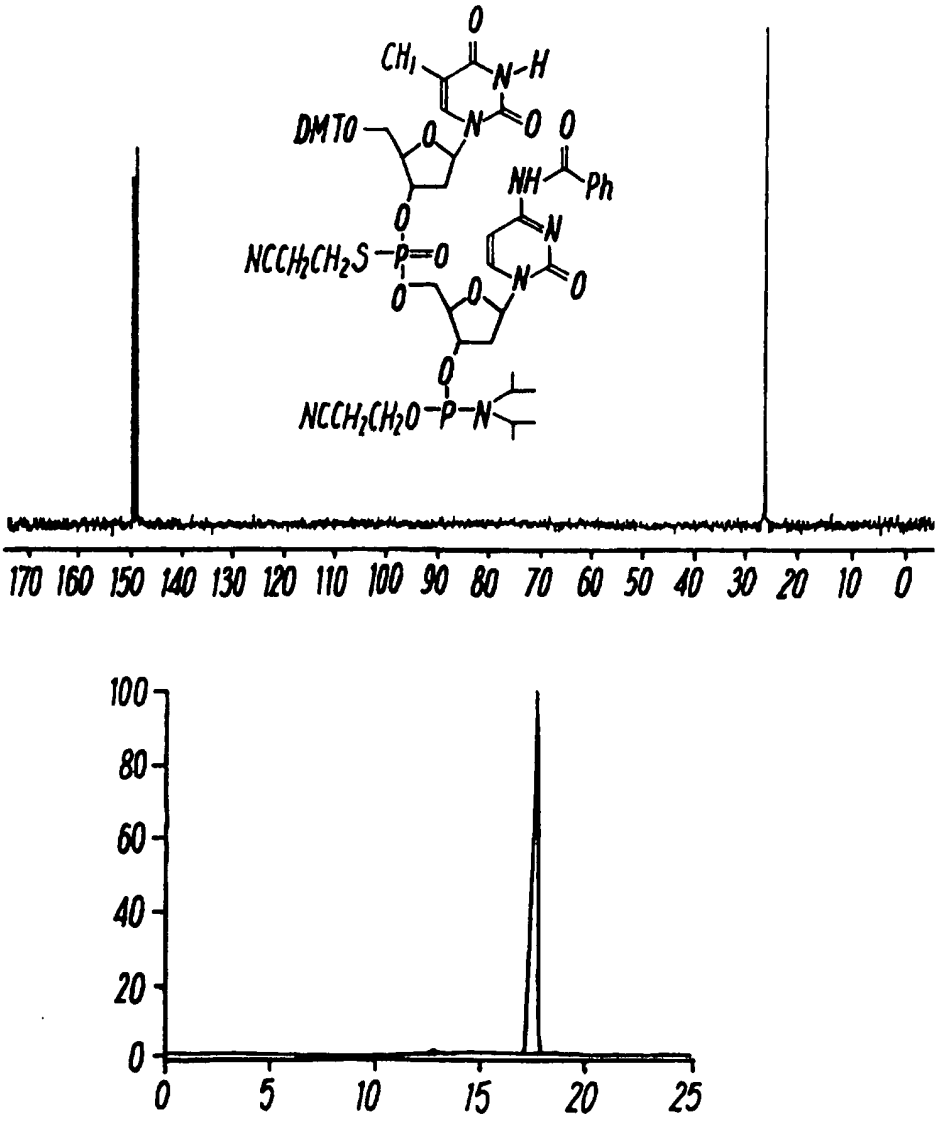

CH3
N-H
DMTO
NH
Ph
NCCH2CH2S
NCCH2CH2O
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
100
80
60
40
20
0
0 5 10 15 20 25

FIG. 1

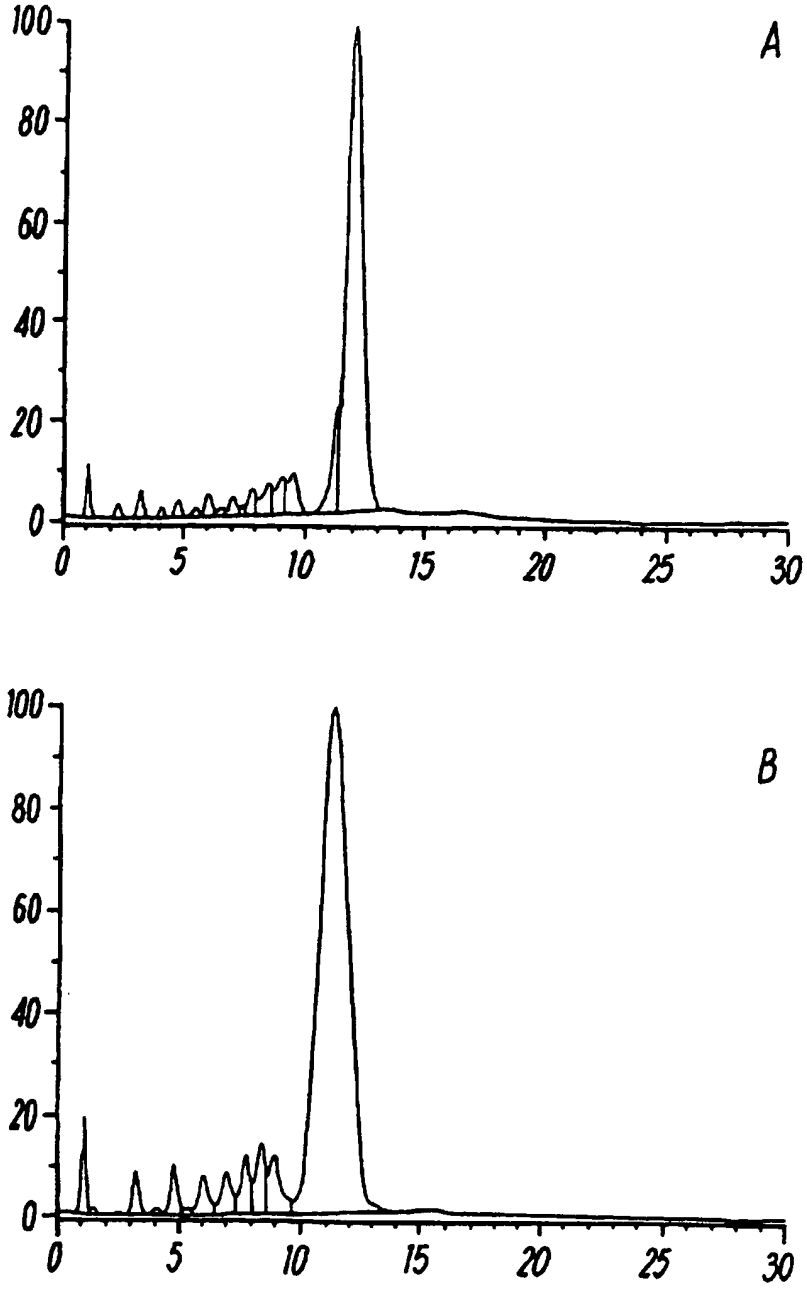

A
100
80
60
40
20
0
0
5
10
15
20
25
30
B
100
80
60
40
20
0
0
5
10
15
20
25
30

FIG. 2

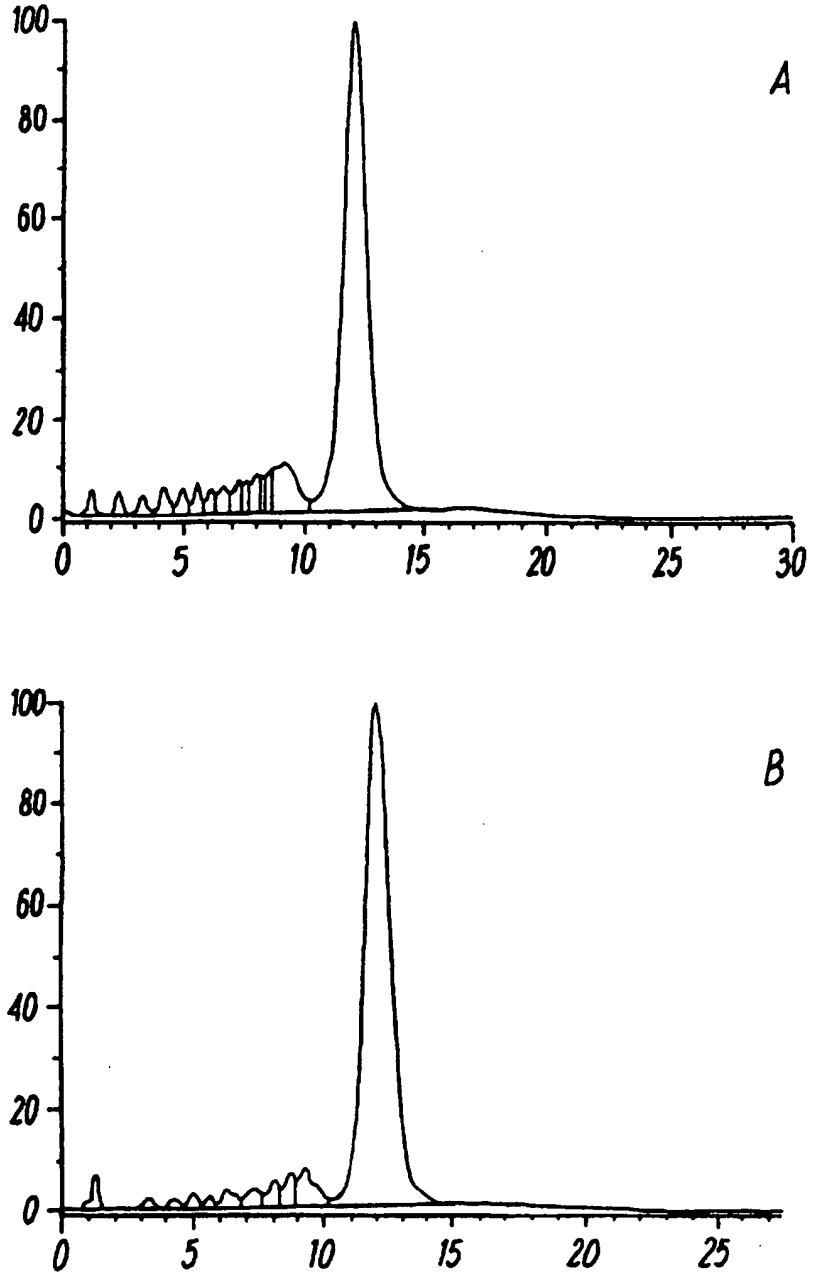


FIG. 3

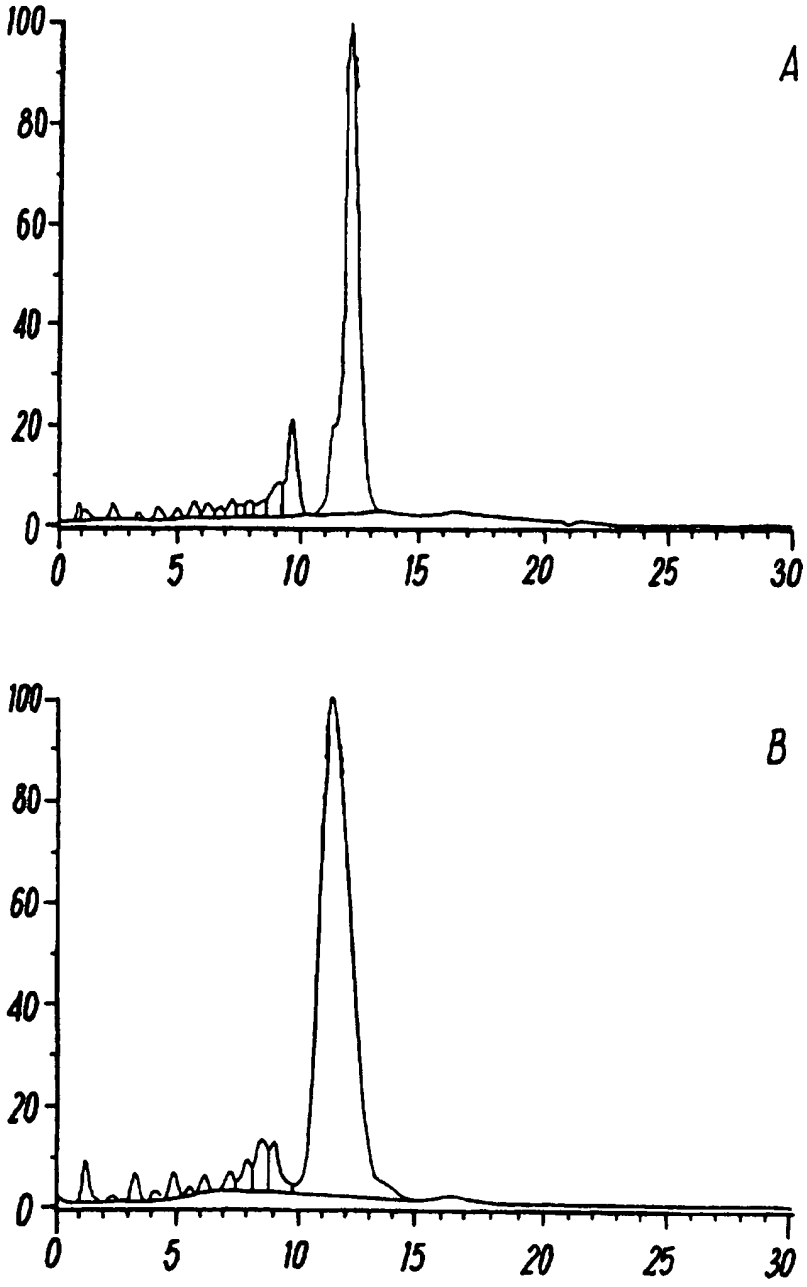


FIG. 4

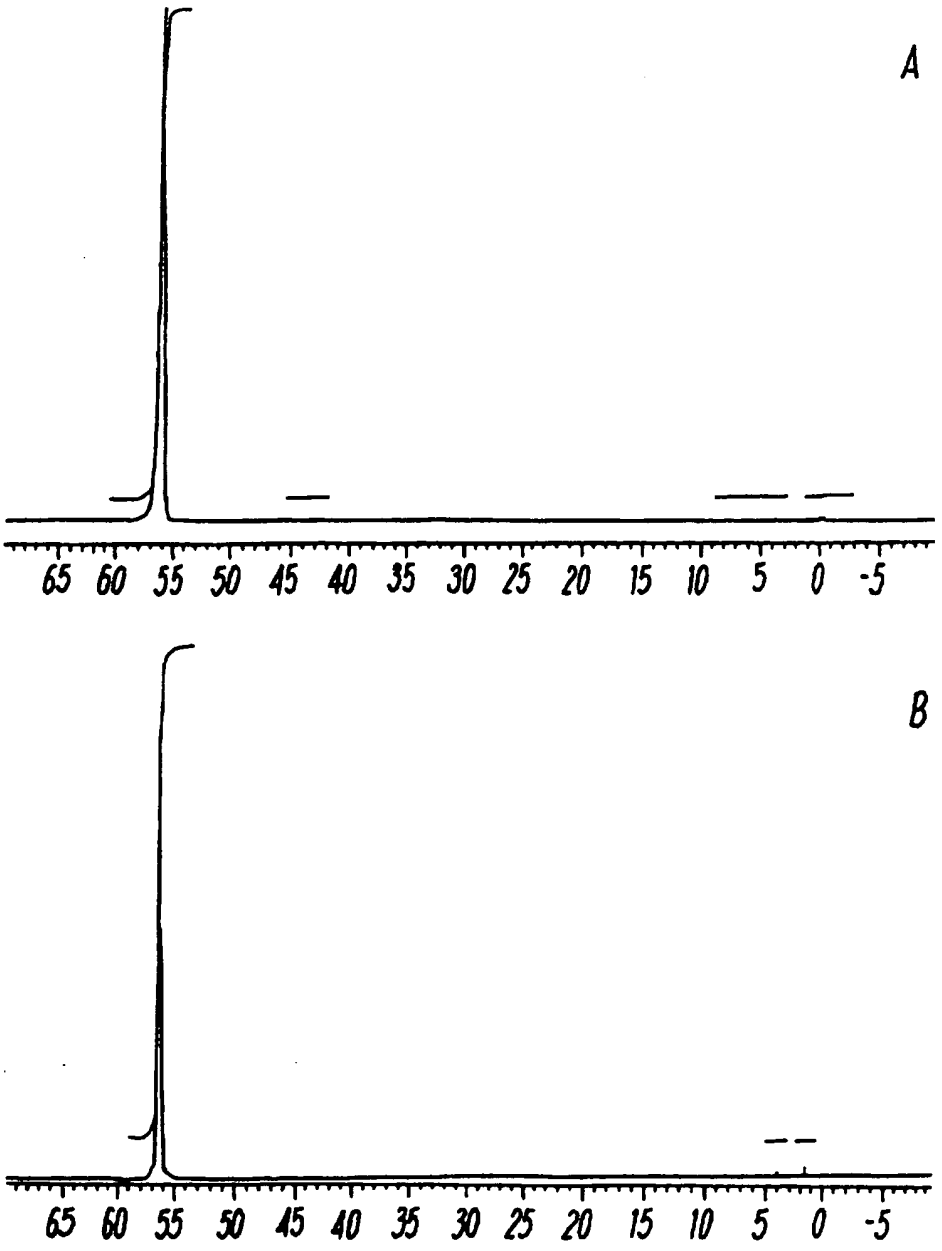

A
65 60 55 50 45 40 35 30 25 20 15 10 5 0 -5
B
65 60 55 50 45 40 35 30 25 20 15 10 5 0 -5

FIG. 5

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/GB 97/00327

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEOSIDES & NUCLEOTIDES, vol. 11, no. 9, 1 January 1992, pages 1621-1638, XP000564715 ZBIGNIEW J LESNIKOWSKI: "THE FIRST STEREOCONTROLLED SYNTHESIS OF THIOOLIGORIBONUCLEOTIDE: (RPRP)- AND (SPSP)-UPSUPSU" see the whole document ---	1-11
A	WO 95 32980 A (ISIS PHARMACEUTICALS INC ;RAVIKUMAR VASULINGA (US); COLE DOUGLAS L) 7 December 1995 see the whole document ---	1,4
A	WO 95 14029 A (BECKMAN INSTRUMENTS INC) 26 May 1995 see the whole document ---	1,4

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 1997	24-06-1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Moreno, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/GB 97/00327

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 668 777 A (CARUTHERS MARVIN H ET AL) 26 May 1987 see the whole document ---	1,4
A	US 5 151 510 A (STEC WOJCIECH J ET AL) 29 September 1992 see the whole document -----	1,4

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

formation on patent family members

Interr nal Application No
PCI/GB 97/00327

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9532980 A	07-12-95	US 5571902 A	05-11-96
		AU 2657095 A	21-12-95
		EP 0766688 A	09-04-97
WO 9514029 A	26-05-95	US 5616700 A	01-04-97
US 4668777 A	26-05-87	US 4415732 A	15-11-83
		US 4973679 A	27-11-90
		AU 551324 B	24-04-86
		AU 8199782 A	30-09-82
		CA 1203237 A	15-04-86
		EP 0061746 A	06-10-82
		JP 1638762 C	31-01-92
		JP 57176998 A	30-10-82
		JP 63028439 B	08-06-88
		JP 1515844 C	24-08-89
		JP 63065677 B	16-12-88
		JP 63179889 A	23-07-88
US 5151510 A	29-09-92	EP 0524942 A	03-02-93
		WO 9116331 A	31-10-91
		US 5292875 A	08-03-94